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Development of an ILTV vaccine - production and characterization of pseudotyped virus-like particles

Inaugural-Dissertation

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1. Summary

1.1 Summary

Basis of this work was the development of a new vaccine for the protection of chickens against infectious laryngotracheitis (ILT), which is caused by the infectious laryngotracheitis virus (ILTV). For this purpose, virus-like particles (VLPs) displaying particular glycoproteins of ILTV were generated.

In Switzerland, ILT is among the notifiable diseases and immunization is not permitted. Live attenuated vaccines pose a certain risk of mutating or recombining with field strains resulting in new pathogenic variants. Furthermore, it is hard to differ between vaccine- and field-strains. In contrast, VLPs are neither infectious nor replication competent and they offer the opportunity to present antigens in high density at their surface and thereby evoke an immune response.

In this work, VLPs based on the murine leukemia virus (MLV) were used. The ILTV glycoproteins gB, gC, gD, and gJ were fused to the transmembrane domain of the epidermal growth factor receptor. This allows the incorporation into the plasma membrane and thus the presentation at the particle surface. The VLPs were generated *via* transfection of two expression vectors (1. ILTV glycoprotein, 2. MLV gag) into 293T cells and various techniques for their characterization were established.

Further work will show, whether the developed VLPs confer a robust protection against ILTV infection.

Keywords: Virus-like particle, infectious laryngotracheitis, Vaccine

1.2 Zusammenfassung

Basis dieser Arbeit war die Entwicklung eines neuen Impfstoffs zum Schutz gegen die infektiöse Laryngotracheitis (ILT) beim Huhn, die durch das infektiöses Laryngotracheitis Virus (ILTV) verursacht wird. Dazu wurden pseudotypisierte virusähnliche Partikel hergestellt (VÄP), die auf ihrer Oberfläche ein bestimmtes Glykoprotein des ILTV tragen.

In der Schweiz ist ILT eine meldepflichtige Erkrankung und das Impfen ist verboten. Bei attenuierten Lebendimpfstoffen besteht ein Risiko, dass durch Mutation oder durch Rekombination mit einem Wildtypvirus ein neues pathogenes Virus entstehen könnte. Auch die Unterscheidung zwischen Impfstämmen und Wildtypviren ist schwierig. VÄP hingegen sind weder infektiös noch replikationsfähig und bieten den Vorteil, ein bestimmtes Antigen in hoher Dichte auf ihrer Oberfläche zu präsentieren und dadurch eine Immunantwort hervorzurufen.

In dieser Arbeit wurden VÄP basierend auf dem murinen Leukämievirus (MLV) verwendet. Die ILTV Glycoproteine gB, gC, gD und gJ wurden an die Transmembrandomäne des epidermalen Wachstumsfaktor-Rezeptors fusioniert. Dieser ermöglicht eine Inkorporation in die Plasmamembran und damit die Präsentation auf VÄP. Durch Transfektion von zwei Expressionsvektoren (1. ILTV Glykoprotein, 2. MLV gag) in 293T Zellen wurden die pseudotypisierten VÄP hergestellt und verschiedene Methoden zu deren Charakterisierung etabliert.

Weitere Arbeiten werden zeigen, ob die VÄP einen ausreichenden Schutz gegen eine ILTV-Infektion bieten.

Stichworte: Virusähnliche Partikel, Infektiöse Laryngotracheitis, Impfstoff

2. Introduction

2.1 General biology of infectious laryngotracheitis virus

Infectious laryngotracheitis virus (ILTV) is classified as a member of the family *Herpesviridae* in the subfamily of *Alphaherpesvirinae* (Fig. 1). Besides the Psittacid herpesvirus 1 it is the only member of the genus *Iltovirus*. ILTV is taxonomically identified as Gallid herpesvirus 1 (GaHV-1) (Roizman, 1982). It is possible to distinguish several genetically different classes of ILTV by a combination of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Up to now, nine classes have been identified (Blacker et al., 2011).

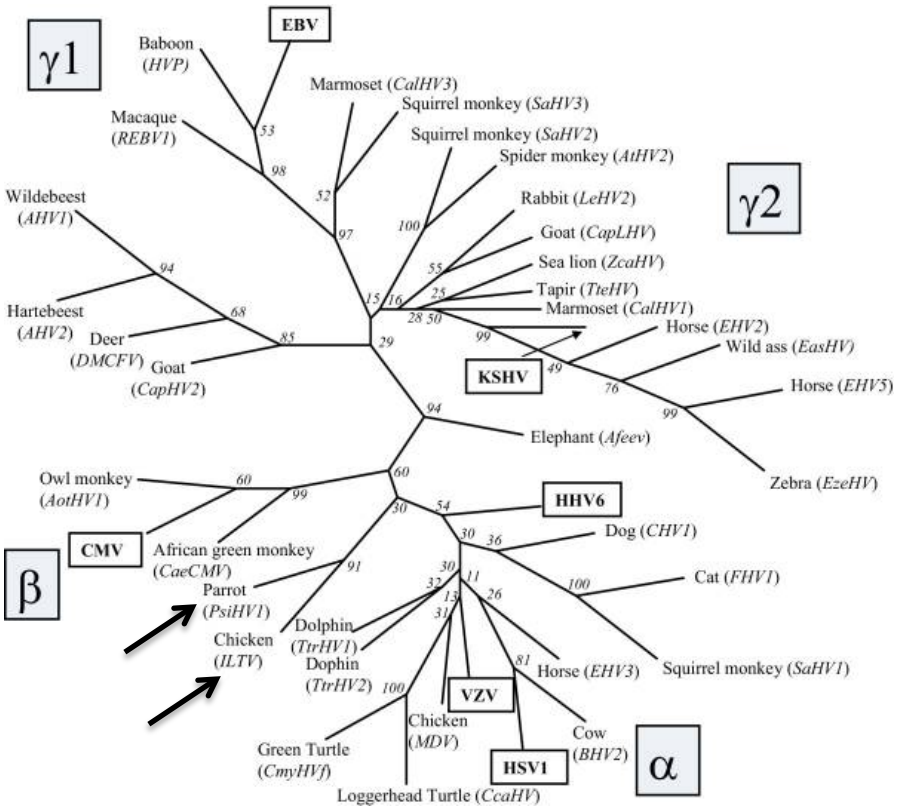

Figure 1: Phylogenetic analysis of DNA polymerase sequences from different herpesvirus species. (Rose, 2005). Arrows indicate the two members of the genus *Iltovirus*.

The ILTV genome is a linear double-stranded DNA molecule (Swayne et al., 2013). Early electron micrographs of GaHV-1 infected chicken embryo cells demonstrated the presence of nucleocapsids with icosahedral symmetry (Cruickshank et al., 1963; Watrach et al., 1963).

These nucleocapsids measure 80-100 nm in diameter. The whole virus particle with its irregular envelope, surrounding the nucleocapsid and the tegument proteins varies in diameter between 200-350 nm (Fuchs et al., 2007). The envelope has characteristic surface projections identified as surface glycoprotein spikes (Fuchs et al., 2007).

Although not analysed in detail GaHV-1 seems to initiate infection like other herpesviruses (Fuchs et al., 2007) i. e., by attachment to its target cell followed by fusion of the viral envelope with the host cell plasma membrane. The nucleocapsid is released into the cytoplasm and transported to the nuclear membrane. Viral DNA is released from the nucleocapsid and migrates into the nucleus through nuclear pores. Transcription and replication of viral DNA occurs within the nucleus (Guo et al., 1993). Newly synthesized viral DNA is cleaved into unit lengths and encapsulated into nucleocapsids. These DNA-filled nucleocapsids acquire an envelope by migration through the inner lamellae of the nuclear membrane. Enveloped particles then migrate through the endoplasmic reticulum and accumulate within vacuoles in the cytoplasm and are finally released by cell lysis or by vacuolar membrane fusion and exocytosis (Guo et al., 1993; Mettenleiter et al., 2002).

2.2 Infectious laryngotracheitis (ILT): hosts, clinical signs and relevance

Chickens are the natural hosts of ILTV. Since the early description of the disease, ILT virus infections have been reported in pheasants, pheasant-chicken crosses, peafowls and young turkeys (Crawshaw et al., 1982; Winterfield, & So, 1968). Subclinical infections with seroconversion were also reported in ducks (Yamada et al., 1980).

Birds of all ages can be affected. The most characteristic signs of the disease can be observed as early as three weeks of age (Dufour-Zavala, 2008). Disease signs generally appear 6-14 days following natural exposure (Jordan, 1966). Infected birds show dyspnea with expectoration of blood-stained mucus and moderate to severe conjunctivitis, respiratory rales, watery eyes, swelling of infraorbital sinuses, mucoid tracheitis and persistent nasal discharge. Furthermore, ILT leads to decreased egg production and reduced weight gain (Swayne et al., 2013), which in turn results in significant economic losses in the poultry industry worldwide (Shan-Chia & Giambrone, 2012). Besides suffering of the animals, this is the main reason why ILT is a major threat for the poultry industry.

2.3 Prevention and control of ILTV infection

In Switzerland, ILT is reckoned among the notifiable animal diseases. Since 1987 various outbreaks were reported. Notably, most of them were registered in fancy fowl. Upon verification of infection the affected livestock has to be culled, because surviving animals are silent carriers of ILTV. The so-called latency is a characteristic of herpesviruses. ILTV resides in the trigeminal ganglion after acute infection (Williams et al., 1992). Certain stress inducing conditions can provoke the recurrence of virus shedding, which can result in the infection of so far healthy animals (Hughes et al., 1989). Since there is no treatment against ILT, the only way to prevent infection would be an efficient vaccination. But even though various vaccines have been developed, immunization is not allowed in Switzerland and the disease is mainly controlled by prevention through biological safety measures (Albicker-Rippinger et al., 1998). However, in most parts of the world chickens are vaccinated which bares certain risks (see 2.3.2).

2.3.1 ILT vaccines

Shortly after ILT was first described in 1925 (May & Tittsler, 1925), immunization of chickens was achieved by inoculating birds with virulent virus *via* the cloaca (Brandly et al., 1934). This is considered the first effective vaccine developed for a major avian viral disease (Guy & Garcia, 2008). Subsequently, attenuated live vaccines were developed by consecutive passages of virulent virus in cell cultures (tissue culture origin, TCO) (Gelenczei & Marti, 1965) or in embryonated chicken eggs (chicken embryo origin, CEO) (Samberg et al., 1969). These vaccines confer a robust protection against ILT infection, but they also bear a risk of reversion to virulence (see 2.3.2).

To improve safety of ILTV immunization, various viral vector vaccines have been developed during the last years. Now available are Innovax ILT® (Merck/ MSD Animal Health Intervet) and Vectormune® FP LT (Ceva, Lenexa, Kansas, USA). Innovax ILT® is based on a turkey herpes virus (HVT), which has been genetically modified to express ILT glycoproteins I and D. It is approved for *in ovo* administration of 18-day-old embryos as well as for subcutaneous application in day-old chickens. The immunity against ILT is supposed to last for at least 60 weeks. Vectormune® FP LT is also a recombinant live virus based on a Fowl Pox virus, which has been genetically modified to express ILT glycoprotein B and the membrane protein UL34. The vaccine is approved for wing web application of eight-week-old chickens.

Two other methods for immunizing chickens against ILTV were published, but not further developed into commercial vaccines. One method relies on the intraperitoneal application of affinity purified ILTV glycoprotein B (gB) in four-week-old chickens. This approach resulted in a protection of 83% of the chickens, as determined by the absence of ILT antigen in the trachea (York & Fahey, 1991). The other published experimental vaccination is also based on the antigen gB, but in this case a bicistronic DNA vector encoding gB and interleukin 18 (IL18) was administered into the muscle of three-week-old chickens. 80% of the challenged chickens didn't show any clinical signs, and PCR for ILTV in tracheal swab was negative (Chen et al., 2010).

2.3.2 Advantages and disadvantages of currently available ILT vaccines

To date, the most common vaccines against ILT are attenuated live vaccines. They are easy to apply – usually *via* drinking water or coarse spray – and confer a robust protection. However, live vaccines can induce latent infections, and occasional virus shedding is possible. Because of their ability to replicate, attenuated live vaccines can revert to more virulent variants, which was already shown for CEO but not for TCO vaccines (Guy et al., 1991). Furthermore, recombination between vaccine- and wild-type-strains, or between various circulating vaccine strains was observed. The latter was shown for some viruses, which were isolated during ILT outbreaks in 2008 and 2009 in Australia. The respective isolates were assigned to the new classes 8 and 9 (Blacker et al., 2011). In a later study, it was shown that these two new classes emerged most likely through interspecies recombination between the co-circulating Australian-origin (Poulvac®Laryngo SA2 and A20, Pfizer) and European-origin (Nobilis®ILT, Serva strain, Intervet) vaccines (Lee et al., 2012). Even though such recombination events seem to be rare, they can result in a fitness-advantage and cause a serious threat for poultry.

Another issue of attenuated live vaccines is the difficult differentiation between vaccine and field strains. To circumvent this issue, several approaches with genetically modified viruses were published. An early attempt focused on the thymidine kinase (TK), which is known to be a virulence factor in other herpesviruses. Upon deletion of the TK gene the mutant ILTV showed a reduced virulence and induced protection against virulent ILTV *in vivo* (Han et al., 2002). Other ILTV mutants were generated by deletion of UL0, glycoprotein J (gJ/US5), UL47 or glycoprotein C (gC/UL44). Even though most of these mutations led to reduced

titers, none of them was essential for replication of the virus. *In vivo* experiments showed a lower pathogenicity of the deletion mutants, and a sufficient protection against infection with wild type strains (Veits at al., 2003; Fuchs et al., 2005; Helferich at al., 2007; Pavlova et al., 2010). Furthermore, these mutant ILTVs enable a serological differentiation between vaccinated and infected birds.

The so far safest vaccines are the recombinant vector vaccines. The big advantage is that they may not establish a latent infection. One of the commercially available vector vaccines is based on a turkey herpesvirus (Innovax ILT®) and is applicable by *in ovo* administration. The drawback of this vaccine is the limited protection effect. However, according to the manufacturer it should last for at least 60 weeks, which is sufficient for most commercial implementations. According to Vagnozzi and coworkers (2012) the protection achieved with Innovax ILT® is almost as good as with the attenuated live ILT vaccines. In contrast, the other recombinant vector vaccine, based on a fowlpox virus (Vectormune® FP LT), was less efficient. Furthermore, it is only recommended for wing web application, which poses problems for mass application. Both vaccines have in common that they are based on replicating viral vectors. Even though both of them are considered as apathogenic for chicken, these are still replicating viruses and thus bear a certain risk of mutation and unforeseeable consequences.

2.4 Virus-like particles (VLPs)

In contrast to attenuated live vaccines, so called subunit vaccines (SUVs) only consist of specific components of a pathogen (Murray, 1988). Mainly surface proteins of various pathogens have been used for the development of SUVs. But compared to attenuated live vaccines, the immunogenicity of SUVs is usually much lower. To overcome this issue various application techniques have been used, e.g. administration of adjuvants, booster injections or high doses of SUVs. Another way to improve the low immunogenicity was the development of virus-like particles (VLPs). Structural proteins of viruses tend to self-assemble into particles that mimic the morphology of the cognate pathogen. As viruses, VLPs can be divided into two groups: enveloped and non-enveloped VLPs. The later ones only consist of the viral components, which are sufficient to self-assemble into a particle. By fusion of the coding sequences of additional, e.g., immunogenic antigens to those of the self-assembling pathogen part, chimeric VLPs can be generated (Fig. 2A, 2C). Some non-enveloped VLP

vaccines are already licenced. Examples are GenHevac B®, Engerix-B®, and Recombivax H® (against hepatitis B virus; HBV) and Cervarix ® and Gardasil® (against human papilloma virus; HPV). Enveloped VLPs are more complex. Besides the viral core proteins they also contain envelope proteins, which are incorporated into the surrounding membrane. These incorporated envelope proteins can either be from the same or from a different pathogen than the core proteins (Fig. 2B, 2D). And since these envelope proteins do not need to be covalently fused to another viral protein, the enveloped VLPs provide more flexibility in terms of generating chimeric VLPs. Nevertheless, both kinds of VLPs display antigenic epitopes in a highly repetitive manner and thus are able to provoke a strong cellular and humoral immune response (Deml et al., 2005; Shen et al., 2013). Besides the display of a single type of antigen per VLP, it is also possible to mix different VLPs for vaccination or to display various antigens on a particular VLP. VLPs are considered as very safe, since they are non-infective and non-replicating.

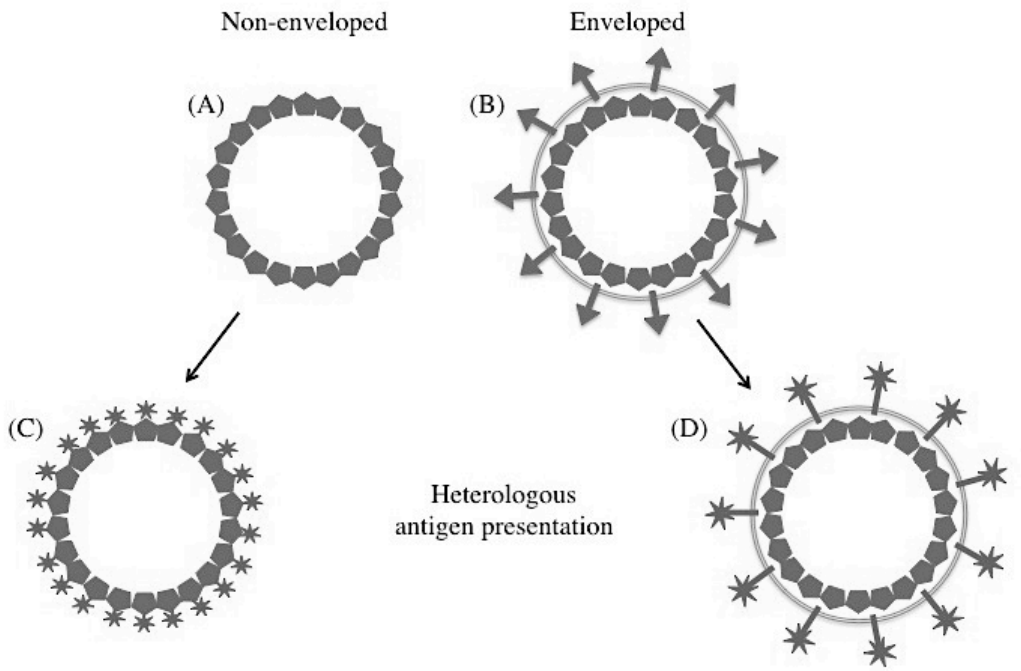

Figure 2: Types of virus-like particles (VLPs). (A) Unmodified non-enveloped or (B) enveloped VLPs. Modified VLPs for presentation of heterologous antigens achieved either by (C) fusion of the respective epitopes to the self-assembling viral proteins or by (D) incorporation of antigens into the particle surrounding membrane.

2.5 Antigen candidates for vaccination against ILTV

Glycoproteins located on the virus surface are major targets for the natural immune response (Ghiasi et al., 1996; Kreijtz et al., 2011) and appear to be highly immunogenic antigens, capable of eliciting both humoral and cell mediated responses (York & Fahey, 1990). Thus, they are promising candidates for potential vaccination approaches.

ILTV potentially encodes 12 glycoproteins, which are conserved among the alphaherpesviruses. But so far, only nine of them have been identified on the protein level. These are gB (Poulsen & Keeler, 1997), gC (Kingsley et al., 1994), gD, gE, gI (Pavlova et al., 2013), gG (Kongsuwan et al., 1993), gJ (Fuchs et al., 2005), gM and gN (Fuchs et al., 2005). Four of them were chosen for the development of a new ILT vaccine and are described in more detail in the following section.

2.5.1 Glycoprotein B (gB, UL27)

This is the most highly conserved herpesvirus structural glycoprotein (Pereira, 1994). Its function in ILTV is not known but it seems to be part of the basic fusion machinery as it was shown for other alphaherpesvirus gB proteins and thus is important for cell entry (Cai et al., 1988). gB is synthesized in the endoplasmatic reticulum where it forms a homodimer and subsequently gets transported to the Golgi apparatus where further modifications and proteolytic cleavage takes place (Poulsen & Keeler, 1997). In contrast to Poulsen & Keeler, (1997) findings, the protein interaction prediction of: www.uniprot.org states that ILTV gB forms homotrimers. This protein is one of the most promising antigen candidates for vaccine development, since gB from various other herpesviruses elicit humoral (Keller et al., 1984) as well as cell-mediated immune (CMI) responses (Zarling et al., 1986). Even though the avian immune system is not comparable with the mammalian system, there is evidence for the existence of a humoral as well as a CMI reaction (Kaiser, 2010). For protection against ILTV infection the CMI seems to be more important (Fahey et al., 1983; Fahey & York, 1990; Honda et al., 1994). So far, several vaccination approaches using gB as an antigen have been published, e.g. application of affinity purified glycoproteins (York & Fahey, 1991), administration of a bicistronic plasmid vector (Chen et al., 2010) and recombinant fowlpox virus or turkey herpesvirus both expressing gB (Tong et al., 2001; Esaki et al., 2013).

2.5.2 Glycoprotein C (gC, UL44)

For most alphaherpesviruses gC is not needed for infection of cells in tissue culture (Robbins et al., 1986) but it was shown that virus binding is increased up to 10 fold in the presence of gC. This might be due to an interaction of this glycoprotein with heparan sulphate on the cell surface (Osterrieder, 1999; Mettenleiter, 1990). Beyond its role in virus attachment, gC is also supposed to be involved in virus egress (Schreurs et al., 1988). It plays a role as virulence factor (Moffat et al., 1998) linked to the fact that it binds to the complement component C3b resulting in immune-evasion (Friedman et al., 1984). ILTV gC was described the first time in 1994. It shows low amino acid homology to other herpesvirus gC proteins and it does not contain a charged extracellular domain, which would mediate binding to surface proteoglycans (Kingsley et al., 1994). An ILTV gC deletion-mutant was tested for its pathogenicity *in vivo*. It was attenuated in chicken, but high doses still led to severe clinical signs in 21% of the infected birds (Pavlova et al., 2010). Promising vaccination approaches against infection were observed with bovine herpesvirus-1 in cattle and duck plague virus in ducks. In both studies, a DNA vaccine encoding the respective gC protein was used (Gupta et al., 2001; Lian et al., 2011).

2.5.3 Glycoprotein D (gD, US6)

This glycoprotein is present in most alphaherpesviruses except in varicella zoster virus (VZV) and Marek disease virus (MDV). In fact, MDV contains a gene encoding for gD but so far it was not detected on the protein level (Tan et al., 2001; Davison, 2010). In other herpesviruses it is necessary for cell binding. Two receptors have been identified that interact with gD. These are nectin-1 and herpesvirus entry mediator (HVEM) (Heldwein & Krummenacher, 2008). gD might also trigger later steps in virus entry, that finally lead to membrane fusion (Lazear et al., 2012). ILTV gD is essential for virus replication and is supposed to act in virus binding and entry (Pavlova et al., 2013). HSV-1 gD was used as a recombinant expressed antigen in vaccination approaches using vaccinia virus as a vector since 1985. Immunized mice were protected against latent herpesvirus infection (Cremer et al., 1985). For vaccination against ILT, two approaches using gD have been published. Both used recombinant viruses expressing gD. One was based on turkey herpesvirus (HVT) and the other on Newcastle disease virus (NDV) (Gimeno et al., 2011; Zhao et al., 2014).

2.5.4 Glycoprotein J (gJ, US5)

gJ is encoded within the unique short (U_S) genome region of ILTV. There are positional homologues in many other herpesviruses, but they hardly share any sequence homology. According to immunoelectron micrographs, the protein seems to be associated with the viral envelope (Veits, et al., 2003). Supporting these data, gJ exhibits characteristics of a membrane protein like N-glycosylation site as well as a membrane anchor signal sequence (Fuchs et al., 2005). But in contrast to many other envelope glycoproteins, gJ is unlikely involved in virus attachment or cell entry but is supposed to play a role during virus egress (Mundt et al., 2011). Even though gJ seems to be highly immunogenic, no neutralizing antibodies were detected so far. A gJ deletion mutant was used as a potential vaccine that would enable serological differentiation between the vaccine and field strains (Fuchs et al., 2005).

2.6 Aim of the study

In the present work, we anticipated to develop a new ILT vaccine based on virus-like particles. Four ILTV glycoproteins (gB, gC, gD, gJ) should be displayed on the surface of VLPs which are derived from the moloney murine leukemia virus (MMLV) (Fig. 3). To achieve the incorporation of the glycoproteins, they were fused to the transmembrane domain of a growth factor which is incorporated into the plasma membrane and thus also into VLPs (Fig. 4).

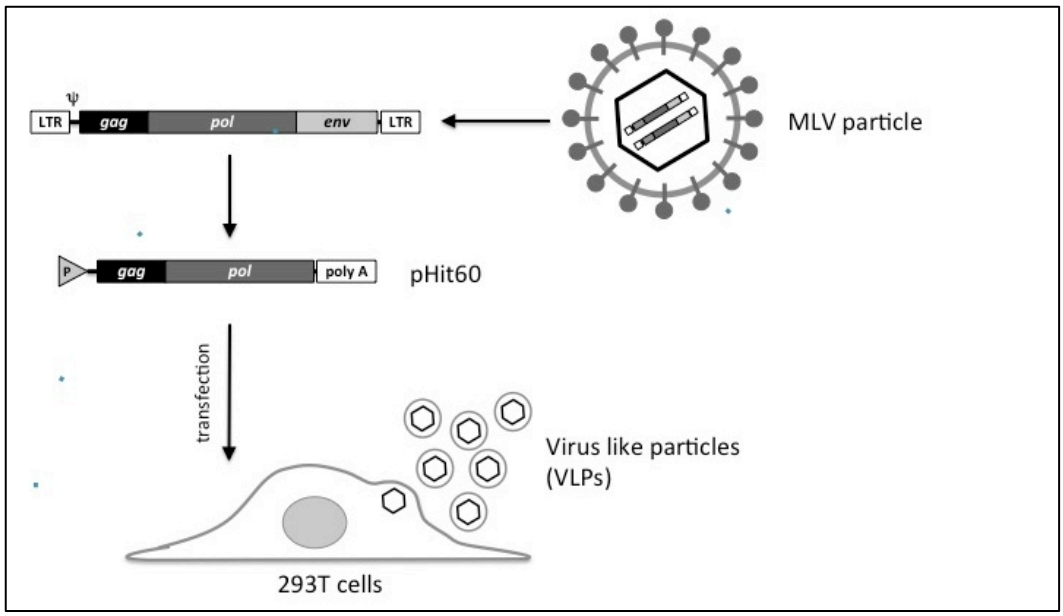

Figure 3: Generation of virus-like particles (VLPs). Upper right: Scheme of a wild type MLV particle. Upper left: Scheme of the viral genome. Middle left: Genome part of MLV sufficient for the formation of VLPs (structural protein gag). Lower part: Upon transfection of the gag encoding plasmid (pHit60) into the producer cell line 293T, virus-like particles will be secreted into the supernatant.

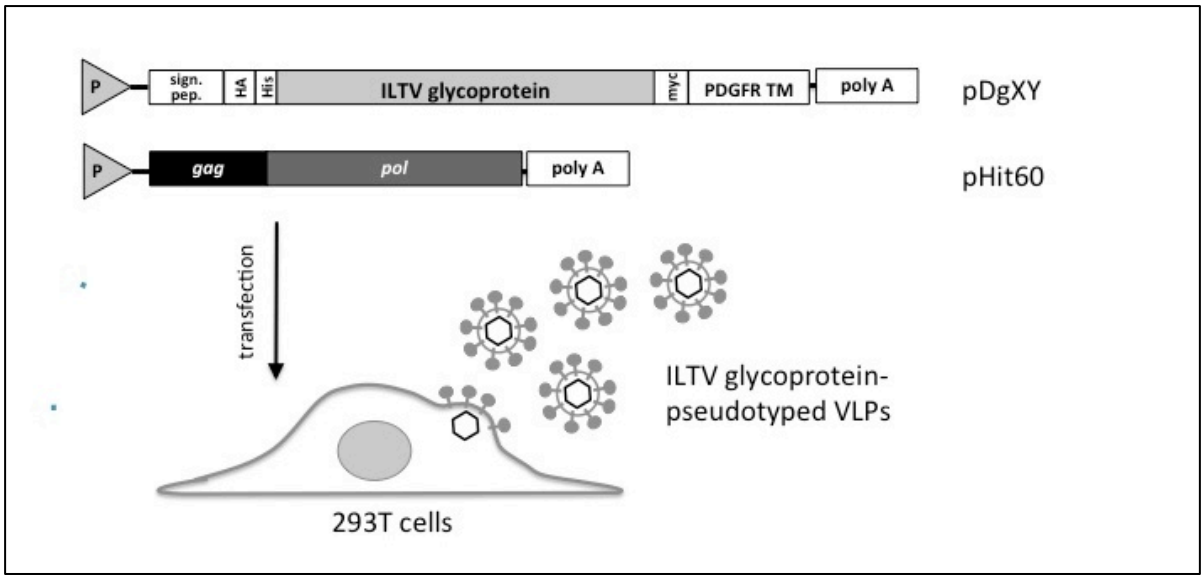

Figure 4: Generation of ILTV glycoprotein displaying VLPs. Upper part: Scheme of the relevant plasmid parts encoding for the ILTV glycoprotein, the transmembrane domain (platelet derived growthfactor receptor transmembrane domain; PDGFR TM) and the tags (HA, His, myc), as well as the VLP structural protein gag encoding plasmid pHit60. Lower part: Upon transfection of both plasmids into 293T cells the VLPs with the incorporated ILTV glycoproteins will be released into the cell supernatant.

3. Materials and Methods

3.1 Cloning of ILTV glycoproteins into an expression vector

To facilitate the display of the ILTV glycoproteins on the VLP surface, they need to be incorporated into the plasma membrane of the VLP producer cell. To achieve this, the respective glycoprotein sequences and fragments thereof were cloned into the plasmid pDisplay™. This plasmid is an expression vector that already contains the murine Ig-κ chain leader sequence, which will direct the fusion protein to the secretory pathway. Furthermore, it contains the platelet derived growth factor receptor (PDGFR) transmembrane domain (TM), which will anchor the protein to the plasma membrane, displaying the protein on the cell surface. For easy detection by Western blot (WB) or immunofluorescence (IF) the influenza virus hemagglutinin (HA) and myc epitopes are encoded on the plasmid as well (a map of the plasmid is shown in Fig. 6). ILTV glycoproteins also contain one or more TM domains. The putative TM domains were excluded from cloning. Only the N-terminal parts that are displayed on the surface of ILTV particles were used for cloning. In Fig. 5 a scheme of the relevant part of pDisplay as well as the various ILTV glycoprotein fragments are shown.

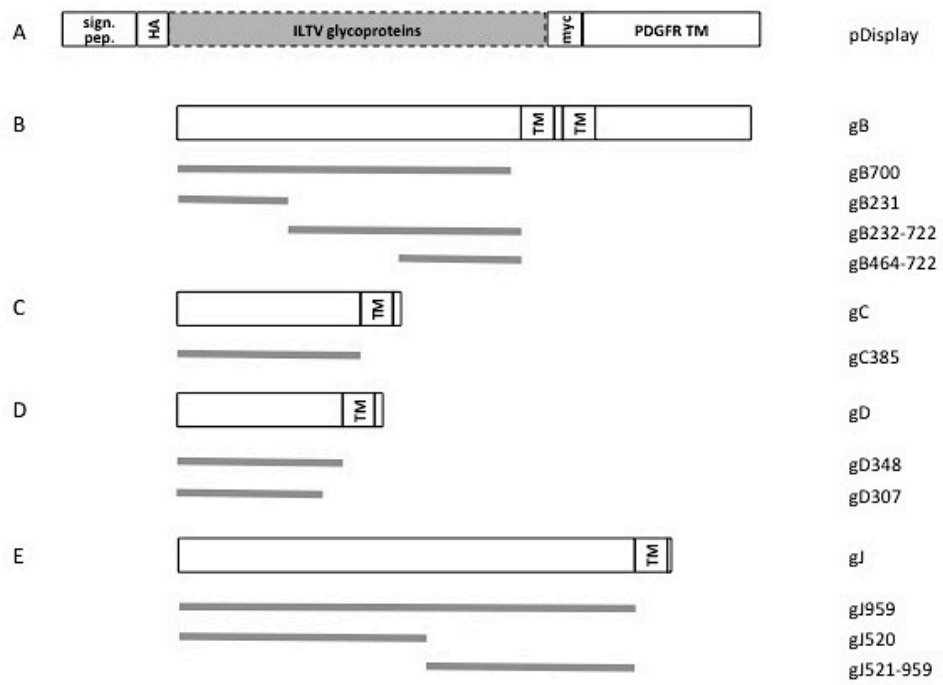

Figure 5: Scheme of pDisplay and the ILTV glycoproteins A. A scheme of the relevant part of the cloning vector pDisplay. B-E. Upper row: Scheme of the respective ILTV glycoprotein containing one or two putative transmembrane (TM) domains. Lower rows:

Position of the various fragments of the glycoproteins that were to be expressed. The numbers behind the respective glycoprotein fragment correspond to the number of amino acids.

The oligonucleotide sequences used for cloning are listed in Table 1 and the reagents used in Table 2.

Oligo-nucleotide name	**Sequence**	**Rest-riction site**
gB1(+)	GTAT**GGCCCAGCCGGCC**CACCATCATCATCACCACGCTAGCGCACAATCCTACATCGCCGTG	SfiI
gB231(-)	GACT**CCGCGG**CCTTCCCTCGATTTCCACAACACAGTCGACCGATGTAG	SacII
gB232(+)	GTAG**GGCCCAGCCGGCC**CACCATCATCATCACCACGCTAGCGCATATCTACAGGCTAGATCTG	SfiI
gB464(+)	GTAT**GGCCCAGCCGGCC**CACCATCATCATCACCACGCTAGCGCATTTGCCATGTTACAATTTG	SfiI
gB722_Sal(-)	GACT**GTCGAC**CCTTCCCTCGATCTTTCCTACTTCTCCAAGAGTGTTG	SalI
gC1(+)	GTAT**GGCCCAGCCGGCC**CACCATCATCATCACCACGCTAGCGCACAGCATCAGAGTACTGCG	SfiI
gC385_Sal(-)	GACT**GTCGAC**CCTTCCCTCGATGACTGCGGGGAATCCTTGCCGCATTG	SalI
gD1_Bgl(+)	GTAG**AGATCT**CACCATCATCATCACCACGCTAGCGCAGACCGCCATTTATTTTTG	BglII
gD307_Sal(-)	GACT**GTCGAC**CCTTCCCTCGATGGGGAGGGCGGCAGGATTTTC	SalI
gD349_Sal(-)	GACT**GTCGAC**CCTTCCCTCGATGGAGACGGCATTAGAACTTTTAGTAGTC	SalI
gJ1(+)	GTAT**GGCCCAGCCGGCC**CACCATCATCATCACCACGCTAGCGCAGGGACAATGTTAGTGTTG	SfiI
gJ959(-)	GACT**CCGCGG**CCTTCCCTCGATGAGTGAATACAGCTGCGCCTGAGCAG	SacII
gJ959_Pst(-)	GACT**CTGCAG**CCTTCCCTCGATGAGTGAATACAGCTGCGCCTGAGCAG	PstI
gJ520(-)	GACT**CCGCGG**CCTTCCCTCGATGGTCTGGGCAGGTGCCTCGGTTTCTG	SacII
gJ521_Bgl(+)	GTAG**AGATCT**CACCATCATCATCACCACGCTAGCGCACCGAGCACGATACCCGAG	BglII

Table 1: Oligonucleotides used for cloning. Bold: restriction sites, underlined: 6x His tag

Product	**Source**
10x loading-Buffer for DNA-Agarose Gels	1.5 g Ficoll 400, 2 ml EDTA, 0.5 ml bromophenol blue, at 7 ml H2O
1Kb Plus DNA Ladder	www.lifetechnologies.com, Cat. No. 10488-085
Agarose, LE, Analytical Grade	www.promega.de, Cat. No. V3121
dNTPs (10 mM)	www.qiagen.com, Cat. No. 201900
DreamTaq DNA Polymerase	www.thermoscientificbio.com, Cat. No. EP0701
Ethidiumbromid (EtBr) 98%	www.carlroth.com, Cat. No. 2218.1
ILTV DNA Strain A489	Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany
LB-Agar Plates (incl. 100 μg/ ml Ampicillin) Difco TM	www.bd.com, Cat. No. 215331
NucleoSpin® Gel and PCR Clean-up	www.mn-net.com, Cat. No. 740609.10
pDgB700	www.lifetechnologies.com
Phusion high fidelity polymerase	www.neb.com, Cat. No. M0530S
Plasmid pDisplay (see Fig. 6)	www.lifetechnologies.com, Cat. No. V660-20
pHit60	Soneoka et al., 1995
Primer	www.microsynth.ch
QIAGEN Plasmid Maxi/Mini Kit	www.qiagen.com, Cat. Nos. 12123 and 12125
Restriction endonucleases: BamHI, BglI, EcoRI, EcoRV, HindIII, PstI, SacII, SfiI, SspI	www.neb.com
S.O.C Medium	www.lifetechnologies.com, Cat. No. 15544-034
T4 DNA ligase	www. neb.com, Cat. No. M0202S
TAE Buffer	0.4 M TRIS, 0.1 M Na2EDTA pH 8.0, 0.2 M Glacial acetic acid
TOP10 chemically competent E. coli	www.lifetechnologies.com, Cat. No. C4040-03
TOPO® XL PCR Cloning Kit	www.lifetechnologies.com, Cat. No. K4750-10

Table 2: Reagents used for cloning

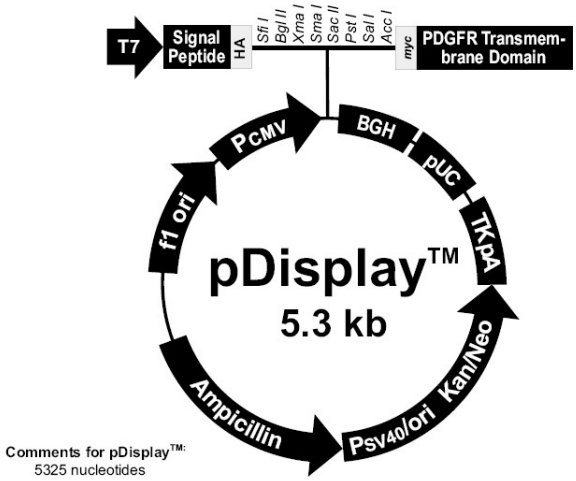

Figure 6: Scheme of pDisplay™. The complete sequence is available on the web site: www.lifetechnologies.com.

3.1.1 Amplification of ILTV glycoprotein sequences

PCR run conditions and the standard PCR reaction mixture are shown in Table 3. The PCR was run in a thermal cycler 2720 (Applied Biosystems, Foster City, CA, USA). The PCR products were purified with NucleoSpin® Gel and PCR Clean-up kit and digested with the respective restriction endonucleases (Table 1) followed by a second purification.
pDisplay was digested with the respective endonucleases.

A

98°C	1’	
98°C	0.5’	25x
45-52°C	0.5’	
72°C	1.5’-3’	
72°C	10’	
4°C	∞	

B

ILTV DNA	50 ng
Dream Taq buffer	5 µl
dNTPs (10mM)	2 µl
Primer (+) (10 µM)	2 µl
Primer (-) (10 µM)	2 µl
Dream Taq	0.5 µl
Add H2O to total	50 µl

Table 3 A: Cycler program used to amplify the ILTV glycoprotein sequences. Annealing temperature was adjusted to primer length and the elongation time to the length of the PCR product. B: Standard PCR preparation

3.1.2 Ligation

The ligation mixtures of prepared pDisplay vectors with selected digested PCR products as inserts are shown in Table 4. The molecular ratio of vector backbone to insert was 1:3. Ligation mixtures were prepared as indicated in table 4 and incubated over night at 16°C or 10 minutes at 22°C. Preparations without insert, one with and one without T4 ligase, served as negative controls.

	neg. 1	**neg. 2**	**pDgB231**	**pDgB232-722**	**pDgB464-722**	**pDgC385**
vector	50 ng	50 ng	50 ng	50 ng	50 ng	50 ng
insert	---------	---------	20 ng	42 ng	22 ng	33 ng
T4 lig. buffer	1 μl	1 μl	1 μl	1 μl	1 μl	1 μl
T4 ligase	0.5 μl	---------	0.5 μl	0.5 μl	0.5 μl	0.5 μl
Add H_2O to a total volume	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl

	pDgD307	**pDgD348**	**pDgJ520**	**pDgJ521-959**	**pDgJ959**
vector	50 ng	50 ng	50 ng	50 ng	50 ng
insert	28 ng	30 ng	44 ng	37 ng	81 ng
T4 lig. buffer	1 μl	1 μl	1 μl	1 μl	1 μl
T4 ligase	0.5 μl	0.5 μl	0.5 μl	0.5 μl	0.5 μl
Add H_2O to a total volume	10 μl	10 μl	10 μl	10 μl	10 μl

Table 4: Ligation mixtures for ligation of ILTV glycoprotein gene sequences into the pDisplay vector.

3.1.3 Transformation of chemically competent *E.coli*

A 100 μl aliquot of chemically competent bacteria was thawed on ice. The whole ligation mix was added and kept on ice for 45 min. Bacteria were heat-shocked for 45 seconds at 42°C. LB-medium without antibiotics was added and bacteria were allowed to recover for 1 h at 37°C. Bacteria were pelleted and supernatant was removed. The pellet was resuspended in

100 µl LB-medium and subsequently plated on, ampicillin-containing plates. Plates were incubated over night at 37°C.

3.1.4 Isolation of DNA from competent *E. coli*

To isolate plasmid DNA from bacteria the respective bacterial colony was transferred from the agar plate into 5 ml LB-medium containing ampicillin (100µg/ml). Bacteria were allowed to grow at 37°C for about 12 hours while shaking. DNA was either extracted directly from the 5 ml culture using the Qiagen Plasmid Mini Kit, or bacteria were further cultivated over night in 200 ml antibiotic containing LB-medium at 37°C while shaking and plasmid DNA was extracted using the Qiagen Plasmid Maxi Kit. Concentration and purity of the isolated plasmid DNA was determined using the NanoDrop spectrophotometer (Witec AG, Littau, Switzerland)

3.1.5 Colony PCR and control restrictions enzyme analyses

To check the cloning product for the correct insertion of the ILTV glycoprotein sequence, whole bacterial colonies were subjected to a PCR. Primers are listed in Table 5. RedTaq® ReadyMix™ and the same cycler program was used as mentioned in 3.1.1 except that the initial step at 98°C was prolonged to 10 minutes. Plasmids were isolated from bacterial colonies that were positive in the PCR and were digested with the endonucleases listed in Table 5. Clones that showed the predicted band pattern upon separation on an agarose gel, were sent for sequencing.

	pDgB231	**pDgB232-722**	**pDgB464-722**	**pDgC385**
Primer (+)	gB1(+)	gB232(+)	gB464(+)	gC1(+)
Primer (-)	gB231(-)	gB722_Sal(-)	gB722_Sal(-)	gC385_Sal(-)
Restriction endonucleases	HindIII	ScaI	SspI	ScaI
Fragment size	5300 bp 743 bp	4055 bp 2756 bp	4042 bp 1121 bp	4729 bp 1776 bp

	pDgD307	pDgD348	pDgJ520	pDgJ521-959	pDgJ959
Primer (+)	gD1_Bgl(+)	gD1(+)	gJ1(+)	gJ521_Bgl(+)	gJ1(+)
Primer (-)	gD307_Sal(-)	gD348(-)	gJ520(-)	gJ959_Pst(-)	gJ959(-)
Restriction endonucleases	ScaI	BamHI	ScaI	EcoRI	EcoRV
Fragment size	4179 bp 2086 bp	5227 bp 1161 bp	3715 bp 3195 bp	5884 bp 783 bp	5556 bp 2671 bp

Table 5: Primers and restriction endonucleases used for verification of the cloning products

3.2 Tissue culture

Product	**Source**
Cell line: 293 T (human, embryonal kidney)	www.dsmz.de, Cat. No. ACC-635
Cell line: LMH (chicken hepatocellular epithelial cell line)	www.cell-lines-service.de
DMEM (Dulbecco's modified eagle's medium – high glucose)	www.sigmaaldrich.com, Cat. No. D6429
DPBS (10X), no calcium, no magnesium (Gibco®)	www.lifetechnologies.com, Cat. No. 14190-094
Dulbecco's Phosphate buffered saline (PBS)	www.sigmaaldrich.com, Cat. No. D8537
Fetal Bovine Serum, Mesenchymal Cell Qualified, US Origin (Gibco®)	www.lifetechnologies.com, Cat. No. 10270
Opti-MEM® Reduced Serum Medium (Gibco®)	www.lifetechnologies.com, Cat. No. 11058-021
RIPA Buffer	www.sigmaaldrich.com, Cat. No. SC 24948
TransIT®-2020 Transfection Reagent	www.mirusbio.com, Cat. No. MIR 5400
Trypsin-EDTA solution (Gibco®)	www.lifetechnologies.com, Cat. No. T 3924
Waymouth's Medium (Gibco®)	www.lifetechnologies.com, Cat. No. 31220-023

Table 6: Reagents used for tissue culture

3.2.1 Cultivation of cells

LMH cells (Kawaguchi et al., 1987) were grown in Waymouth's Medium supplemented with 10% fetal bovine serum (FBS) and cultivated in a humidified incubator at 38°C with 5% CO_2. Cells were subcultivated twice a week in a ratio of 1:5 or 1:6.

293 T cells were grown in DMEM supplemented with 10 % FBS and cultivated in a humidified incubator at 38°C with 5% CO_2. Cells were subcultivated twice a week in a ratio of 1:10.

3.2.2 Transfection

For immunofluorescence (IF) cells were seeded on cover slips in 24-well plates (LMH 1x 10^5/0.5 ml, 293T 8x 10^4/0.5 ml). For preparation of lysates for Western blot analysis or generation of VLP-containing supernatants, cells were seeded into 6-well plates (LMH 7x 10^5/ ml, 293T 5x 10^5/ ml). 24 h later, cells were transfected using the TransIT-2020 transfection reagent according to the manufacturer protocol. After overnight incubation the medium was carefully changed against medium without FBS. As controls, 293T cells were transfected with pHit60 alone or with one of the ILTV glycoprotein expression constructs alone. 48 h after transfection supernatants (SN) were collected, filtered through a 0,45 μm filter, and concentrated by low speed centrifugation (2600x g, 4°C, 24h). Pellets were re-suspended in PBS and used for further analysis.

In the SN of cells that have been transfected with pHit60 and a glycoprotein expression plasmid, both should be detectable by Western blot analysis. In the SN of cells that have been transfected with pHit60 alone, only gag should be recognizable and in SN of cells that have been transfected only with one of the glycoprotein expression construct, nothing should be detectable (Fig. 7: A, B, C).

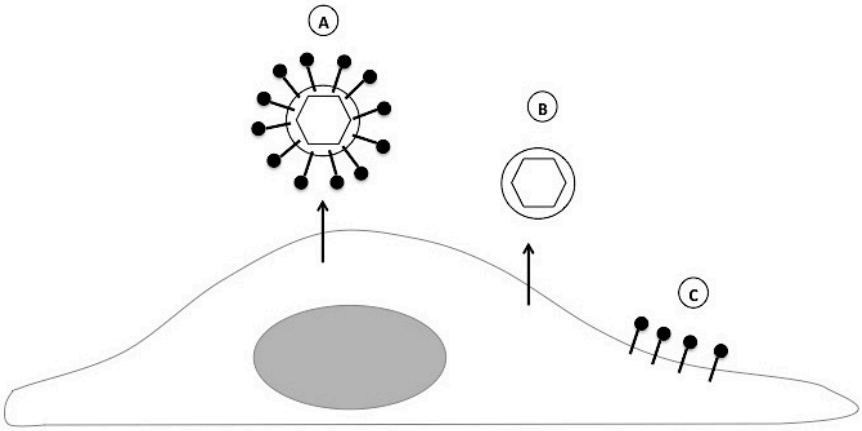

Figure 7: Scheme of the possible outcome after transfection of different plasmids. A: Transfection of pHit60 (encoding MLV gag) and an expression plasmid for one of the ILTV glycoproteins (pDgB700, pDgC385, pDgD307, pDgJ959). B: Transfection of pHit60 alone. C: Transfection of the ILTV glycoprotein expression plasmid alone.

3.2.3 Particle collection and cell lysis

48 h after transfection, VLP-containing supernatants were collected as described in 3.2.2., filtered through a 0.45 μm pore size filter and concentrated by low speed centrifugation at 2600x g at 4°C for 24 h. The supernatant was discarded and each pellet was resuspended in 100 μl PBS. The cells were carefully washed once with PBS. 1 ml fresh PBS was added per well, cells were scraped of and transferred to an Eppendorf tube. Cells were pelleted by centrifugation (10', 10.000x g, 4°C) and subsequently lysed using RIPA lysis buffer.

3.3 Immunofluorescence

Product	**Source**
Anti-HA.11 mouse monoclonal antibody clone16B12	www.covance.com, Cat. No. MMS-01R
Bovine serum albumin (BSA)	www.amresco-inc.com, Cat. No. 8076.3
DAPI	www.lifetechnologies.com, Cat. No. D3571
Donkey anti-mouse IgG-Alexa Fluor® 488 antibody	www.lifetechnologies.com, Cat. No. A-21202

Product	Source
Fluorescent mounting medium	www.dako.com, Cat. No. S3023
Goat serum	www.lifetechnologies.com, Cat. No. 16210-064
Paraformaldehyde	www.carlroth.com, Cat. No. 0335.1
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
RPMI-1640 medium	www.sigmaaldrich.com, Cat. No. R7388
Triton X-100	www.carlroth.com, Cat. No. 3051.3

Table 7: Reagents used for immunofluorescence

Transfected cells were not fixed with paraformaldehyde (PFA) to prevent permeabilization of the plasma membrane, but were cooled down to 4°C to minimize their metabolic activity. Living cells were incubated for 30 minutes at 4°C with a mouse antibody against the HA-tag (diluted 1:300 in RPMI/ 2%BSA). Afterwards, cells were washed and fixed with 3% paraformaldehyde. Upon three times washing with PBS coverslips were incubated with the Alexa Fluor® 488-coupled secondary antibody (diluted 1:1000 in PBS/ 10% goat serum) and DAPI for 45 minutes at room temperature. After three times washing with PBS coverslips were mounted on glass slides and used for imaging.

3.4 Indirect ELISA

Product	Source
Anti-c-Myc-tag rabbit polyclonal antibody	www.genscript.com, Cat. No. A00172-100
Anti-HA.11 mouse monoclonal antibody clone16B12	www.covance.com, Cat. No. MMS-01R
Anti-His mouse monoclonal antibody	www.signalchem.com, Cat. No. H99-63R-25
Anti-ILTV gB goat antiserum	Ceva Biomune, K.Moore Dorsey, Lenexa, KS, USA
Anti-ILTV gC mouse antiserum	Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

Product	**Source**
Anti-ILTV gD rabbit antiserum	Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany
Anti-ILTV gJ mouse antiserum	Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany
Blocking Buffer	5% BSA, 0.1% Tween 20 in PBS
Coating buffer	50mM NaHCO3 pH 9.6
Donkey anti-goat IgG-HRP antibody	www.scbt.com, Cat. No. sc-2020
Goat anti-mouse IgG-HRP antibody	www.scbt.com, Cat. No. sc-2354
Goat anti-rabbit IgG-HRP antibody	www.cellsignal.com, Cat. No. 7074
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4
PBST	0.05% Tween 20 in PBS
Stop buffer	3% HCl
TMB	www.genscript.com, Cat. No. M00078
Triton X-100	www.carlroth.com, Cat. No. 3051.3

Table 8: Reagents and antibodies used for ELISA

Concentrated VLPs were diluted in coating puffer. Triton X-100 was added to a final concentration of 0.5%. 90 μl of each sample was pipetted into a 96 well microtiter plate. After overnight incubation at 4°C, the supernatant was discarded, and the plate was incubated with blocking buffer. After 1 hour at room temperature the supernatant was discarded. Incubation with the first antibody was performed for 1 h at room temperature. After three times washing, incubation with the horseradish peroxidase (HRP) coupled secondary antibody was performed for 1 h at room temperature. The plate was washed three times and 100 μl TMB reagent was added per well. After 30 minutes at room temperature, the reaction was stopped by adding 100 μl stop buffer. The absorbance was measured at 450 nm on a microplate reader Multiskan Ex Primary EIA V.2.3. (Thermo Fisher Scientific, Waltham, MA, USA).

3.5 Western Blot

Product	Source
6x Loading buffer (Lämmli)	60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue
8% SDS polyacrylamide gel	6.7 ml 30% Acrylamid, 6.5 ml lower Tris pH 8.8, 15 µl TEMED, 200 µl APS 10%
Anti-HA.11 mouse monoclonal antibody clone 16B12	www.covance.com, Cat. No. MMS-101R
Anti-MLV Gag (p30) polyclonal rabbit antibody	www.icosagen.com, Cat. No. A1-910-100
Blotting buffer	Cell-buffer, 15% Methanol
Cell-buffer	25 mM Tris-Hydroxy-Methyl-Amoniomethan, 192mM Glycine
Enhanced chemiluminescence (ECL) reagent	0.1 M Tris-HCL(pH 6.8), 125 mg Luminol sodium salt, 35% H_2O_2, Para-Hydroxy Coumarin Acide, Dimethyl Sulfoxide (DMSO)
Fuji Medical X-ray Film 100 NIF	www.fujifilm.com
Goat anti-mouse IgG-HRP antibody	www.scbt.com, Cat. No. sc-2005
Goat anti-rabbit IgG-HRP antibody	www.cellsignal.com, Cat. No. 7074
Non-fat dry milk in TBS	Migros
Ripa-Lysis-buffer	www.piercenet.com, Cat. No. SC 24948
Running-buffer	Cell-buffer, SDS 10%
TBS buffer	19.8 mM Tris, 500 mM NaCl
TTBS buffer	TBS buffer, 0.05% Tween 20
Whatman® Protran® nitrocellulose membranes, pore size 0.45 µm	www.sigmaaldrich.com, Cat. No. 10.401196

Table 9: Reagents and antibodies used for Western blotting

Gel electrophoresis was done in the Mini Protean system (BioRad). Lysates (see 3.2.3) were loaded on 8% SDS polyacrylamide gels and separation was performed following the standard SDS PAGE protocol (Laemmli, 1970). For blotting, filter papers and nitrocellulose membranes were equilibrated in transfer buffer. Transfer was done for 1.5 hours at 20V at room temperature. Membranes were blocked in 5% non-fat dry milk in Tris-Buffered Saline (TBS) for 1 h at room temperature. Incubation with the primary antibody was done over night

at 4 °C under constant shaking. Membranes were washed three times with TTBS for 10 minutes each. Incubation with the secondary antibody (coupled to horseradish peroxidase) was done for 1 hour at room temperature. After washing three times with TTBS for 10 minutes each, detection was done by incubation with ECL reagent for 5 minutes. For visualization, membranes were exposed to Fuji Medical X-ray Film 100 NIF and films were developed using the Agfa Curix 60 (Schenk AG, Hettlingen, Switzerland).

3.6 Immuno gold labelling and electron microscopy

Product	**Source**
Anti-ILTV gJ mouse antiserum	Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany
Bovine serum albumin (BSA)	www.amresco-inc.com, Cat. No. 9048-46-8
Goat anti-mouse IgG gold 10 nm	www.sigmaaldrich.com, Cat. No. G7652
Goat serum	www.lifetechnologies.com, Cat. No. 50197Z
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
Uranylacetate acid	www.polysciences.com, Cat. No. 21447-25

Table 10: Reagents and antibodies used for immuno gold labelling

Concentrated VLPs were spotted on copper grids precoated with parlodion/carbon. After adsorption for 2 minutes, excess buffer was soaked off and grids were washed four times with PBS/10% goat serum for 1 minute. Grids were incubated with the anti-ILTV gJ antibody, diluted 1:50 in PBS/1% goat serum for 30 minutes at room temperature. Grids were washed with PBS/10% goat serum 5 times for 2 minutes and subsequently incubated with the secondary gold-conjugated antibody diluted 1:50 in PBS/0.1% BSA for 30 minutes at room temperature. After washing twice with PBS/0.1% BSA and 4 times with H_2O for 1 minute, grids were treated with 3% uranyl acetate for 20 seconds. Excess fluid was removed and grids were air dried before imaging with the transmission electron microscope (TEM).

4. Results

4.1 Cloning of ILTV-glycoprotein-expression constructs

4.1.1 Control endonuclease digestions of the final cloning products

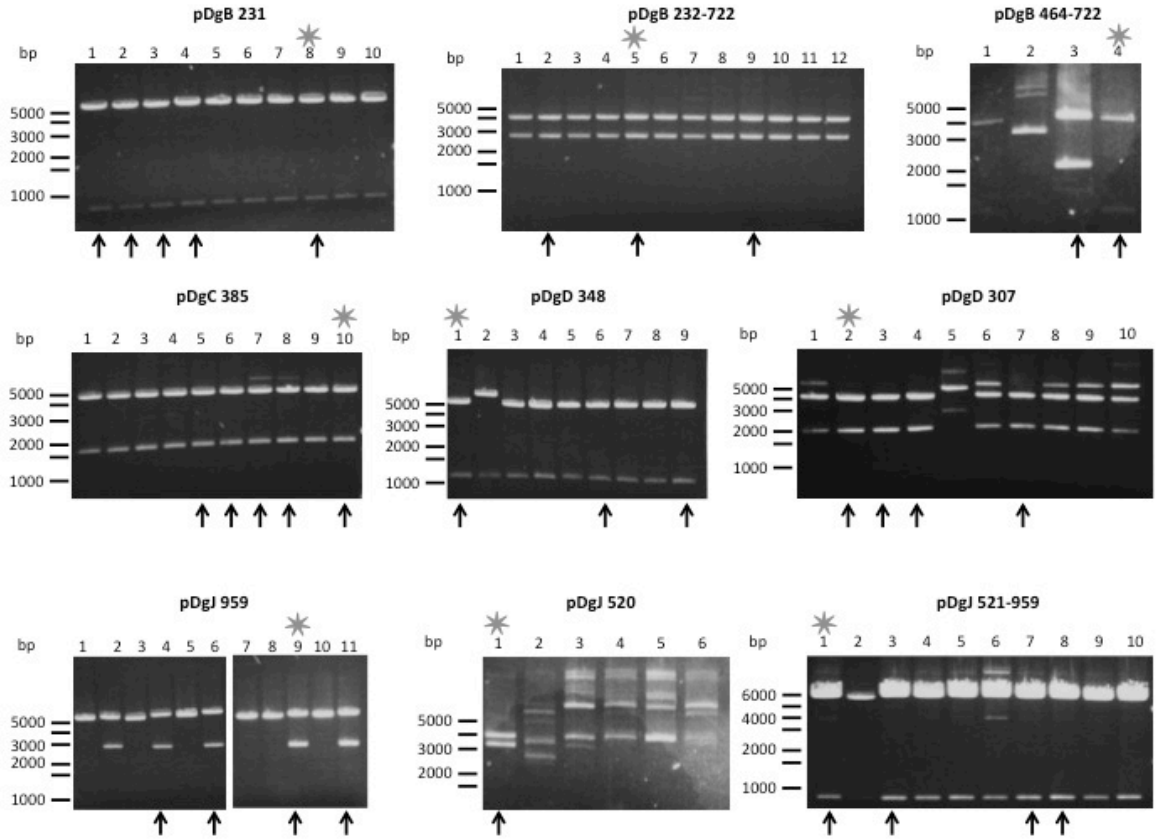

Figure 8: Restriction pattern of the different ILTV glycoprotein expression plasmids. The final cloning products (indicated above the picture) were cleaved with the respective restriction endonucleases and the fragments were separated by gel electrophoresis in a 1% agarose gel supplemented with ethidium bromide. Samples that are marked with an arrow (↑) were sent for sequencing and the one marked with an asterisk (✶) were proven to be correct and were used for further experiments.

4.2 Expression of ILTV glycoproteins

4.2.1 Intracellular expression of ILTV glycoproteins

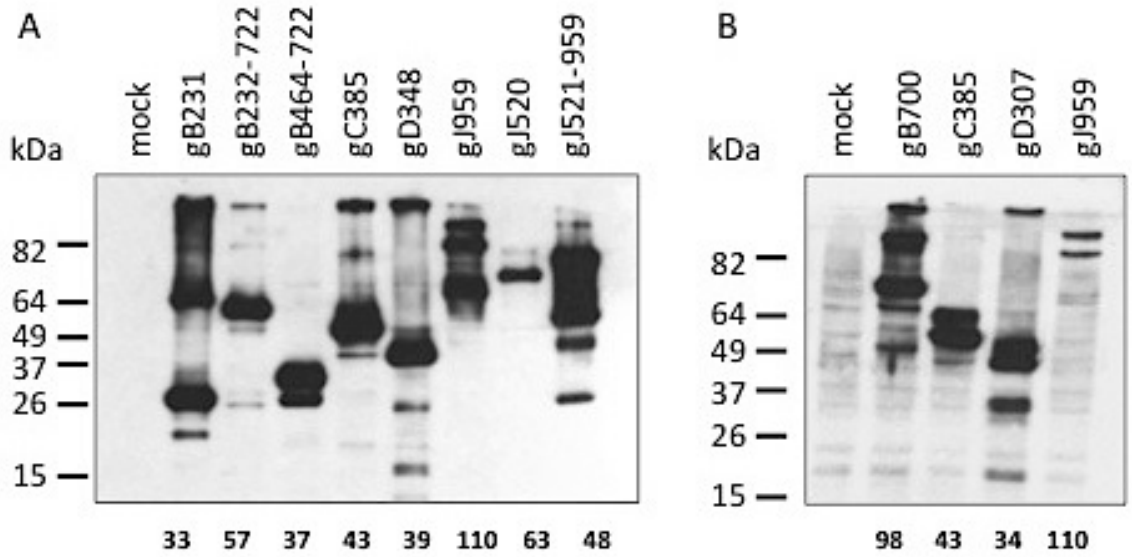

Figure 9: Western blot analysis of the expression of ILTV glycoproteins in 293T cells using anti HA-antibodies. A: Western blot of 293T cells transfected with pDgB231, pDgB232-722, pDgB464-722, pDgC385, pDgD348, pDgJ959, pDgJ520, pDgJ521-959. B: Western blot of 293T cells transfected with the plasmids that were used for further experiments (pDgB700, pDgC385, pDgD307, pDgJ959).

All proteins could be detected (Fig. 9). Most samples showed more than one band. But in all cases the most prominent band corresponded with the predicted molecular weight. For further experiments only constructs that contained the whole surface part were used. These are gB700, gC385, gD307, gJ959 (Fig. 5). Even though gD307 is 41 amino acids (aa) shorter than gD348, it was decided to use this construct, since gD348 is not expressed on the cell surface (data not shown).

4.2.2 Surface expression of ILTV glycoproteins

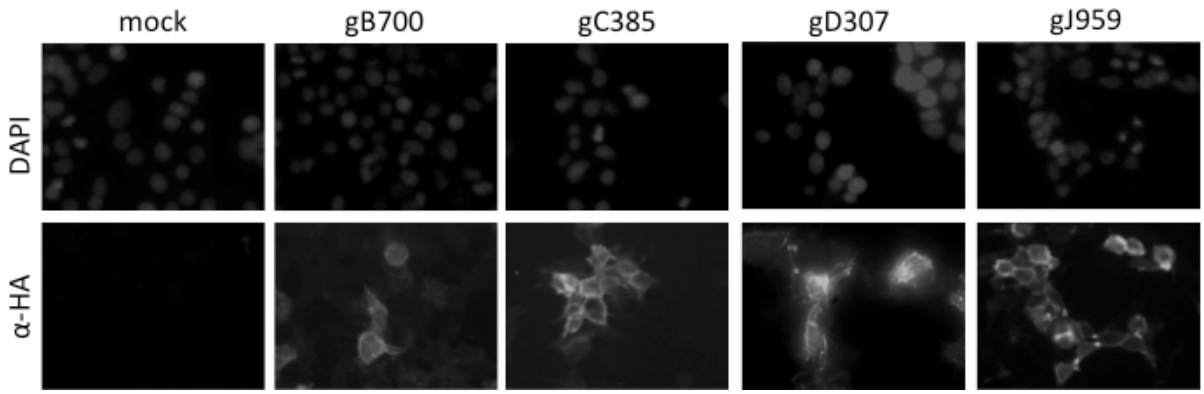

Figure 10: Immunofluorescence analysis of the surface expression of ILTV glycoproteins. Upper row: DAPI. Lower row: anti-HA antibody.

The four glycoproteins gB700, gC385, gD307, gJ959 were detected on the cell surface of transfected 293T cells.

4.3 Expression of pseudotyped virus-like particles (VLPs)

4.3.1 VLP detection by Western blot analysis

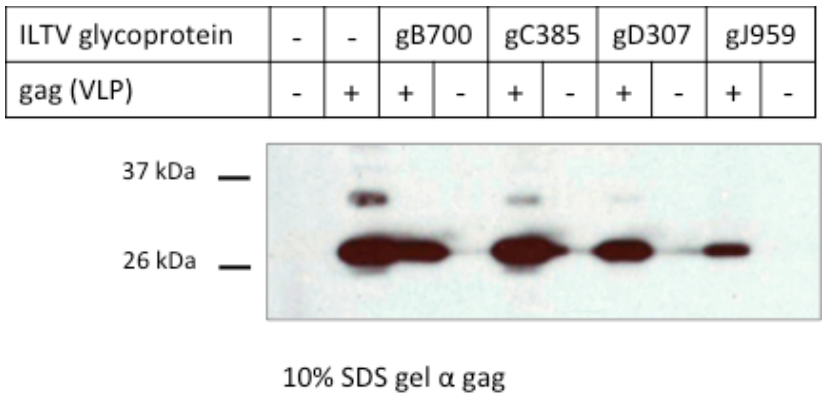

Figure 11: Western blot of SN of transfected 293T cells. Cells were transfected either with a plasmid encoding for the MLV gag protein and/or with an expression construct for the ILTV glycoprotein. VLPs were detected by using an antibody against MLV gag.

MLV gag was detectable in SNs of cells that have been transfected with pHit60, indicating the formation and secretion of VLPs.

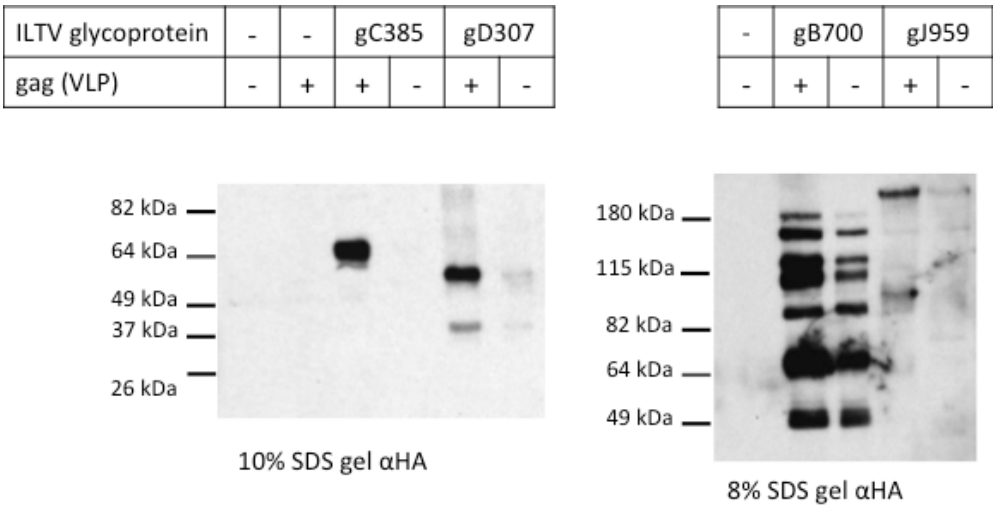

Figure 12: Western blot of SN of transfected 293T cells. Cells were transfected either with a plasmid encoding for the MLV gag protein and/or with an expression construct for the ILTV glycoprotein. The ILTV glycoproteins were detected by using an antibody against the HA-tag.

Only in SN of cells that have been transfected with pHit60 and pDgC385, gDC385 was detected by Western blot. In the case of gD307 or gJ959, also in SN of cells that have been transfected only with the ILTV glycoprotein expression constructs, a slight band is visible. Strong bands appeared for SN of cells that have been transfected exclusively with pDgB700. The bands in the samples which were transfected with pDgB700 and pHit60 were only slightly more intense (Fig. 12).

4.3.2 VLP detection by ELISA

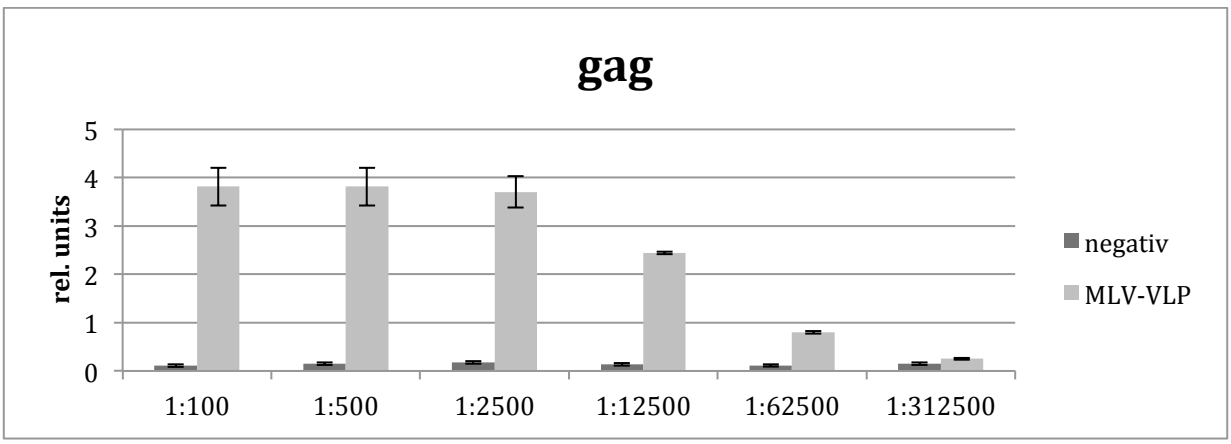

Figure 13: ELISA from supernatants containing VLPs . 293 T cells were transfected with pHit60 alone. Detection with an antibody against MLV gag.

Using the MLV gag antibody enabled the detection of VLPs in concentrated SN of transfected cells. This allows the relative quantification of VLPs.

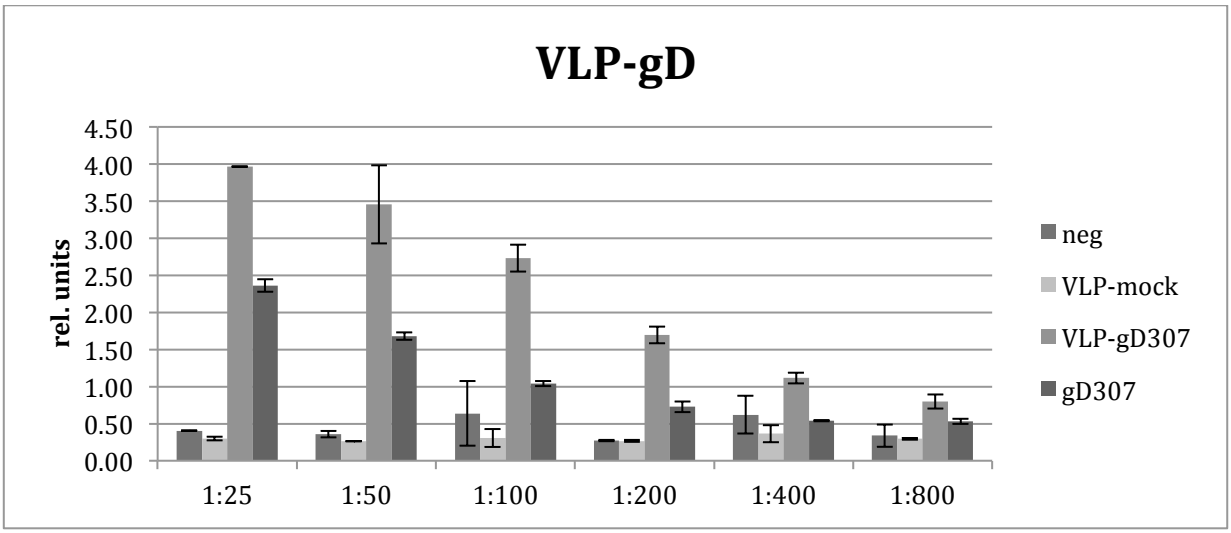

Figure 14: ELISA from supernatants containing gD307 pseudotyped VLPs. 293 T cells were transfected with pHit60 and pDgD307. Detection with an antibody against ILTV gD.

gD307 was detected in SN of VLP-gD307 but also in SN of cells that were transfected with pDgD307 alone.

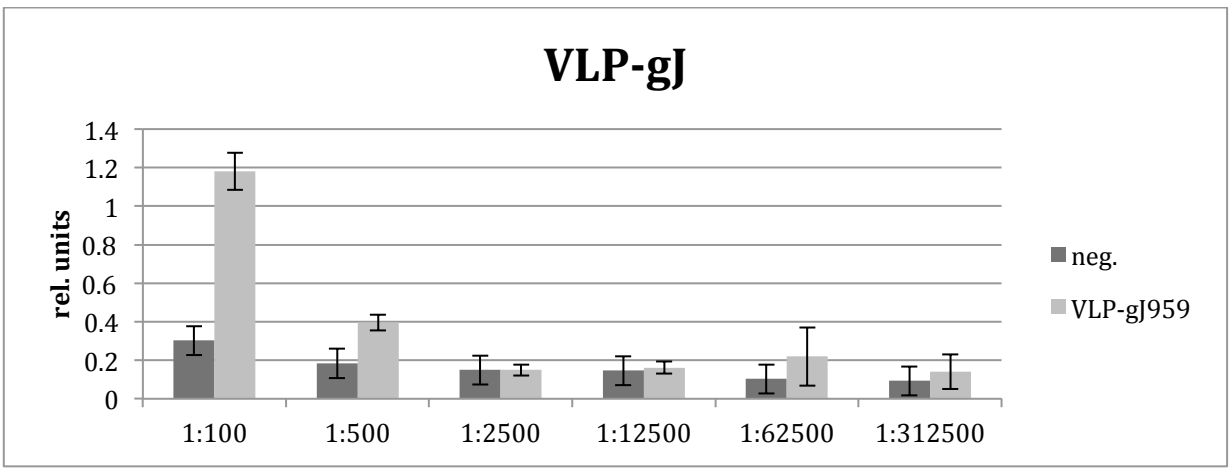

Figure 15: ELISA from supernatants containing gJ959 pseudotyped VLPs. 293 T cells were transfected with pHit60 and pDgJ959. Detection with an antibody against ILTV gJ.

gJ959 was detected in the SN of 293T cells that have been transfected with pHit60 and pDgJ595.
ILTV glycoproteins C and B could not be detected in ELISA (data not shown).

4.3.3 VLP detection by electron microscopy

Using an antibody against ILTV gJ, gJ959 was detected in close proximity to the VLPs, indicating a successful incorporation of the ILTV glycoprotein into the MLV derived VLP.

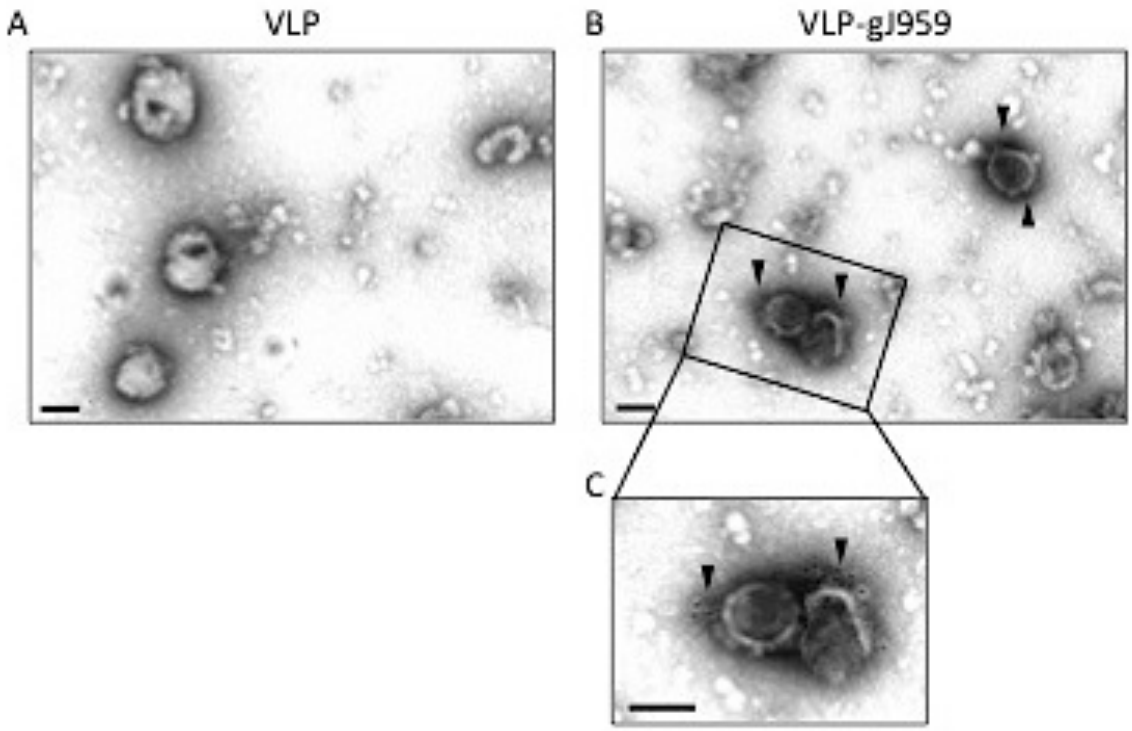

Figure 16: Electron micrographs of immunogold labelled VLPs pseudotyped with gJ959.
A. SN of 293T cells only transfected with pHit60 (VLP). B, C. SN of 293T cells that have been transfected with pHit60 and pDgJ959 (VLP-gJ959). Arrowheads indicate immunogold particles (scale bar 100 nm).

5. Discussion

In this study the basis for a new vaccination approach against ILT in chickens was established. Virus-like particles (VLPs) based on the murine leukemia virus (MLV), were generated that display various glycoproteins of ILTV on their surface.

Expression of all generated ILTV encoding plasmids was verified by Western blotting. All constructs of this study could be detected. Most of the glycoproteins differed slightly from the predicted size and/or showed additional bands, which might be due to the existence of various splice and cleavage products or modifications of the respective proteins. Poulsen & Keeler, (1997) made similar observations in their study. They infected chicken embryonic kidney (CEK) cells with the USDA challenge strain of ILTV. Upon immunoprecipitation with an ILTV gB antiserum, samples were analysed by polyacrylamide gel electrophoresis and revealed two bands with a size of 110 kDa and 58 kDa. Further analyses involving various glycosylation inhibitors showed band shifts to reduced sizes, which led to the final conclusion that gB is expressed as a precursor that will assemble into homodimers or -trimers. On its way through the Golgi it is modified by several glycosylations and finally is proteolytically cleaved to form two disulphide-linked entities, which have the same molecular mass but differ in their modifications (Poulsen & Keeler, 1997). Our results are not directly comparable to this study. Instead of infecting chicken cells with the USDA challenge strain, we used expression constructs encoding various parts of gB and transfected these into the human cell line 293T. The closest to the wild type gB is gB700, which contains the first 700 N-terminal amino acids (aa) of in total 882 aa. The predicted size is 98 kDa. We detected two major bands with sizes of about 100 kDa and 70 kDa and two minor bands with about 65 kDa and 50 kDa. These multiple bands are most likely also due to proteolytical cleavage and glycosylation processes.

The construct gB231, encompassing the first 231 aa, revealed two major bands of about 65 kDa and 28 kDa. The predicted size was 33 kDa. The detected 65 kDa band might be due to a disulfide bond linked version of the small protein. The construct gB232-722 has a predicted size of 57 kDa. In our Western blot a band of about 60 kDa was detected. For the construct gB464-722, which has a predicted size of 37 kDa, two bands were detected. One of about 35 kDa and one with 26 kDa.

In a study published by Pavlova and colleagues Western blot analysis of ILTV-A489 infected CEK cells revealed a single 60 kDa band when using a gC specific antibody. In our study, Western blot analysis of 293T cells transfected with gC385 showed two bands of about 52 kDa and 60 kDa. That the bands were larger than the predicted weight of 43 kDa could be explained by N-glycosylations like it was described for ILTV gC before (Fuchs et al., 2005). The appearance of two bands cannot be explained. In this study, an antibody detecting the HA-tag was used but in further experiments the antibody against ILTV gC will be applied.

Another study by Pavlova and colleagues characterized ILTV gD. Upon infection of CEK cells with ILTV-A489 and Western blot analysis, they detected bands of 70 kDa and 65 kDa. In virus particles only the 70 kDa version could be detected, indicating that this is the mature and functional version of gD. The 65 kDa form of gD was speculated to be an immature, degraded, or differentially processed functional form of gD. The size difference compared to the calculated mass of 48.5 kDa was explained by possible phosphorylations, since no glycosylations were detected (Pavlova et al., 2013). In this study gD348 was transfected into 293T cells and lysates were used for Western blotting. One major band of about 40 kDa was detected and two minor bands of 30 kDa and 16 kDa. In later experiments it was shown that this construct was not expressed on the cell surface. This might be due to an incomplete deletion of the gD transmembrane domain or signal peptide, and thus a possible retention in the endoplasmatic reticulum (data not shown). The construct gD307 only contained the first 307 aa of in total 376 aa. In Western blot analysis of transfected 293T cells three dominant bands with sizes of about 49 kDa, 40 kDa and 30 kDa were detected and a less pronounced band of about 16 kDa.

ILTV gJ is reported to have two splice variants. Fuchs and colleagues (2005) cloned the full genomic gJ gene and the cDNA fragment of the spliced gJ mRNA species. Upon transfection into LMH cells and Western blotting, the spiced gJ revealed a single band at 85 kDa. The full-length construct showed a similar pattern like in cells infected with the wild type ILTV A489 strain, namely four bands with sizes of 200 kDa, 160 kDa, 115 kDa and 85 kDa. Even though the calculated mass would be 106.5 kDa. By using different glycosylation inhibitors, they could show that gJ contains N- and O-glycosylations (Fuchs et al., 2005). In our study, three different gJ expression constructs were generated. The one closest to the wild type is gJ959 containing the first 959 aa of in total 985 aa. Upon expression in 293T cells, the Western blot analysis revealed three bands with sizes of about 100 kDa, 85 kDa and 65 kDa. A fourth

larger band was not detected, but this might be due to the use of 10% SDS polyacrylamide gel. To get a better resolution for bigger proteins, gels with less polyacrylamide will be applied in further experiments. gJ520 only contains the first 520 aa. Since this constructs lack the splice acceptor site, no splice variants could be detected in Western blot analysis. Only a single band of about 70 kDa was visible. The difference to the predicted size of 63 kDa might be due to glycosylations. For gJ521-959 various bands were detected. This was not expected, since this construct contains the splice acceptor but not the splice donor site. Our hypothesis is that this part of the protein forms dimers or is modified in other ways.

For further characterisations, only the glycoprotein-constructs gB700, gC385, gD307 and gJ959 were used. These ones only lack their own TM domain and the C-terminal part, but contain the whole N-terminal region. To test whether the glycoproteins are expressed on the cell surface, immunofluorescence of transfected and non-permeabilized cells was performed. Upon transfection of 293T cells with the glycoprotein expression constructs alone or in combination with the packaging construct encoding for gag, the SN were analysed by Western blotting to check the presence of VLPs with incorporated glycoproteins. All tested glycoproteins could be detected. This implies a successful incorporation of the glycoprotein into the VLP. If transfected alone, a very dim band for gD and gJ was visible, which might be due to a few non-incorporated glycoproteins. In contrast, if gB was transfected alone, the detected bands were only slightly less pronounced compared to samples of SN of cells transfected with gB and gag. This indicates the presence of free gB in the supernatant. A possible cleavage by extracellular proteases is likely to be excluded since addition of protease inhibitors did not reduce the detected protein in the supernatant (data not shown).

For quantification of the VLPs enzyme-linked immunosorbent assays (ELISAs) were established. Detection of gag was successful using a polyclonal gag antibody. Unfortunately, the only glycoproteins that could be detected in ELISA were gD and gJ. Detection was possible with specific antibodies against the respective proteins. The gD ELISA did also support the results obtained by Western blot. Traces of gD were detected in SN of cells that were transfected with gD alone, indicating the presence of non-VLP associated glycoproteins. gB and gC were neither recognized by specific antibodies nor by antibodies against the HA-, His-, or Myc-tag (data not shown). Thus further improvements need to be done, to enable detection of gB and gC in ELISA. The quantification of VLPs by gag ELISA is no suitable

measure to evaluate the effectiveness of vaccine production, since it would be more important to know the amount of antigen displayed at the VLP surface.

A third way of characterising the VLPs was immunogold labelling followed by imaging under an electron microscope. So far, only VLPs pseudotyped with ILTV gJ were successfully labelled and imaged. They were stained with an antibody against ILTV gJ, which in turn was recognized by a secondary antibody coupled to a 10 nm gold particle. The microscopy clearly showed gold particles in close proximity to the VLP, indicating incorporation into the VLP-surrounding membrane. The antibodies against the various tags (HA, His, Myc) did not lead to the recognition of the corresponding glycoproteins. Thus, staining conditions will be improved in further experiments and specific antibodies against gB, gC, and gD will be tested.

In conclusion the basis for the establishment of a new ILT vaccine was developed. ILTV glycoproteins can be incorporated into MLV VLPs. Furthermore, various assays were developed for characterisation and quality control of the generated VLPs. Nevertheless, before ending up with the final VLPs that can be used in immunization experiments, several optimizations need to be done. So far, VLPs were generated by transient transfection of the plasmid pHit60 into 293T cells. pHit60 encodes the MLV gag protein but in addition also the retroviral enzymes (reverse transciptase, integrase, protease). For safety considerations it is desirable to use minimal parts of the parental virus. Since the viral enzymes are not needed for the generation of VLPs, an expression-construct for gag alone will be cloned. This can be used to establish a stable cell line for large scale VLP production. Furthermore, VLPs will be generated in chicken LMH cells instead of human 293T cells. This should prevent unwanted immune reactions to the cell derived membrane surrounding the VLPs. For quantification of VLPs respectively the displayed glycoproteins, a reference is required. Thus, the glycoproteins will be purified from transfected cells and used as a standard in ELISA. Moreover, ELISAs for the detection of gB and gC have to be optimized, e.g. by testing different antibodies and/or modifying the conditions (buffers, incubation times, blocking reagent, etc.), and before using the pseudotyped VLPs *in vivo* they need to be purified.

6. References

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